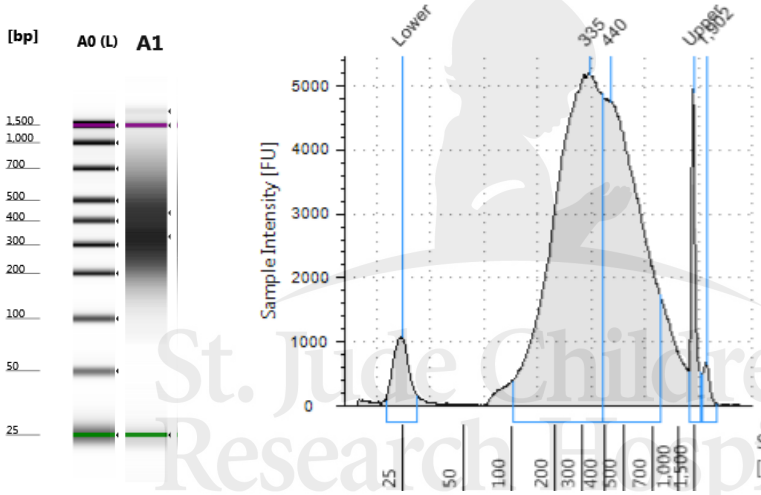
Date: 7/23/14 Experiment #: LG.AbVal.14.1/JM12.14.92
Antibody code: 17; Active Motif 61101

H3K36me3 (abs 17)

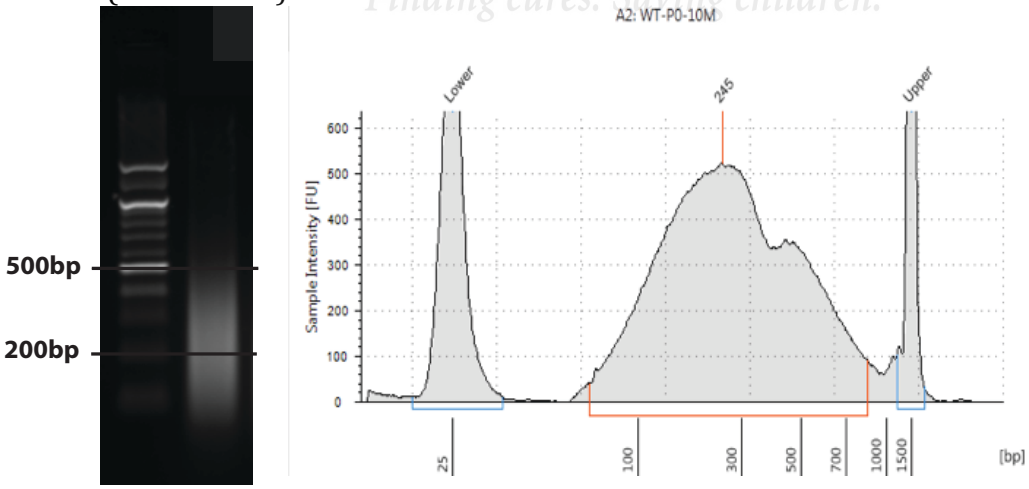
Antibody cat #:	61101
Lot #:	32412003
Company:	Active Motif
Host species:	Rabbit
Date validated:	7/23/14
Validated by Dyer lab member:	Lyra Griffiths; Justina McEvoy
ChIP validated by other:	

Antibody amount per ChIP:	2.5 ug
Number of cells per ChIP:	0.25 million cells (human) 0.5 million (mouse)
Chromatin prep protocol #:	JM12.14.120 (human); ID.1.13.131 (mouse)
Quant-it for human chromatin:	109 ng/µl
Quant-it for mouse chromatin:	77 ng/ul

HUMAN (JM12.14.120_MAST60)



MOUSE (ID.1.13.131)



Date: 7/23/14 Experiment #: LG.AbVal.14.1/JM12.14.92
Antibody code: 17; Active Motif 61101

Level 1: Western Optimization

Purpose:
To test the efficiency and specificity of the antibody in human and mouse cells. This antibody will be used for future IP validation and /or ChIP experiments.

Experiment summary:
5-10 ug of chromatin from human and mouse will be run on a western and immunblotted using the antibody being tested. On the same gel, run the same samples to be immunoblotted for total histone. This will be used for later quantitation.

Procedure: 1-2 days

1) Dilute 28µl of chromatin with 7µl of 5X sample buffer (must add in beta-mercaptoethanol to buffer first) .

2) Heat sample to 95°C for 10 minutes

3) Prepare running buffer and pour into electrophoresis chamber. Place precast gradient gels into the chamber (Biorad TGX 15-well 4-20% (456-1096)). * Remember to remove green strip on the bottom of the gel.

4) Remove comb and rinse out the wells with running buffer.

5) Load 15ul of sample per well. **Remember to run enough lanes to immunoblot for total H3 or total H4.

6) Run gel at 200V (constant volts) for 25 minutes. At this point watch the blue running dye. Continue to let the gel run until the blue dye has reached the bottom. Stop and remove the gel once the blue dye has reached the bottom.

7) Separate the plates and manually remove the blue dye with a razor blade.

8) Place the gel in transfer buffer for 5-10 minutes to equilibrate.

9) Transfer the protein onto 0.2µm nitrocellulose membrane at 100V (constant volts) for one hour.

10) Remove the membrane from transfer apparatus directly into 5mls of Odyssey blocking buffer.

11) Block for one hour. At this point make antibody dilutions using Odyssey blocking buffer. **Remember to immunoblot for total H3 or total H4 for each western. This is important to relatively quantitate each of the H3 marks.

12) Transfer membrane to antibody dilutions and rock over night at 4°C.

13) Wash membrane for 5min with 1% TBST (can find recipe in Dyer lab protocol book). Repeat wash two more times.

14) Transfer membrane to secondary antibody (infrared fluorescent secondary antibody diluted 1:10,000 in Odyssey blocker).

15) Incubate the membrane for 1 hour (covered with foil) at room temperature on the Belly Dancer.

16) Wash membrane for 5min with 1% TBST (can find recipe in Dyer lab protocol book). Repeat wash two more times.

17) Scan membranes on the Odyssey LiCor.

18) Determine the signal for each band using LiCor (please ask Lyra or Justina if you need help).

19) Determine relative amounts of the protein by normalizing to total H3 or H4.

Recipes and antibody dilutions:

<u>RUNNING BUFFER (1 liter)</u>
25mM Tris 3.03g
192mM glycine 14.4g
0.1% SDS 5mls (20%)
Up to 1 L with H2O

<u>TRANSFER BUFFER (1 liter)</u>
25mM Tris 3.03g
192mM glycine 14.4g
methanol 200mls
Up to 1 L with H2O

Primary Antibody	Cat. #	Species	Dilution or µicrograms	µl to add to 5mls of Oddyssey blocker
H3K4me1	Ab8895	Rabbit	1:1000	5
Total H3	Ab10799	Mouse	1:1000	5
Secondary Antibody	Cat. #	Species	Dilution or µicrograms	µl to add to 5mls of Oddyssey blocker
IRDYE680	926-68071	Rabbit	1:10,000	0.5µl
IRDYE800CW	926-32210	Mouse	1:5000	1µl

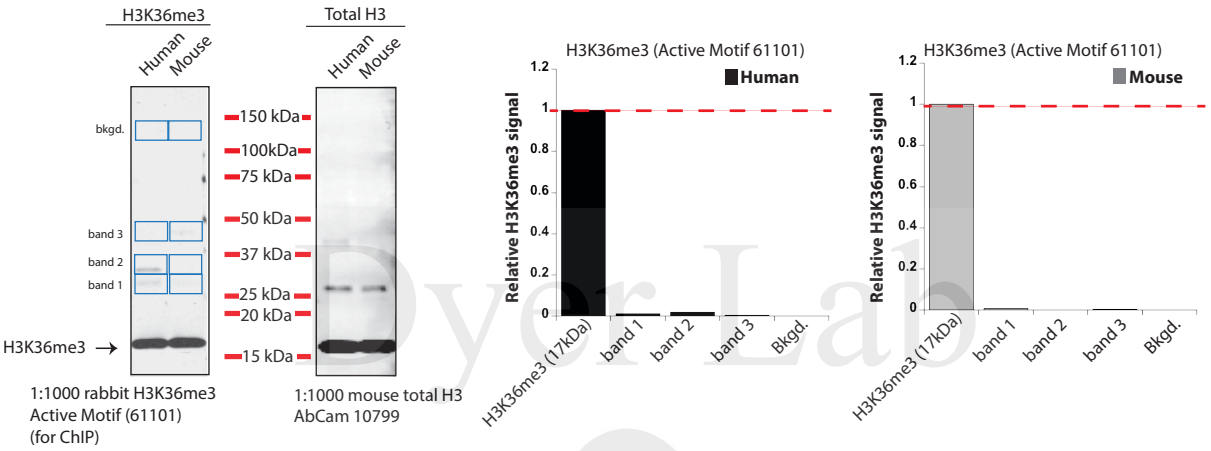
Date: 7/23/14 Experiment #: LG.Ab Val.14.1/JM12.14.105

Antibody 17; Active Motif 61101

Results:

Total protein of human chromatin loaded per lane: 3µg

Total protein of mouse chromatin loaded per lane: 3µg



Species	Band	Relative to human H3K36me3
Human	H3K36me3	1.000
	band 1	0.010
	band 2	0.017
	band 3	0.003
	bkgd.	0.001
Mouse	H3K36me3	1.000
	band 1	0.006
	band 2	0.002
	band 3	0.005
	bkgd.	0.001

Conclusions: (provide yes or no answer)

The background for this antibody in human is acceptable for use in ChIP: YES

Antibody passes validation for the level 1 for ***human***: YES

The background for this antibody in mouse is acceptable for use in ChIP: YES

Antibody passes validation for the level 1 for ***mouse***: YES

Additional notes:

The major proportion of H3K35me3 is at the predicted size of 17kda. This is an acceptable signal for expected band according to ENCODE guidelines where it is recommended that the primary reactive band represents >50% of the signal.

Level 2: ChIP Validation

(proceed if antibody passes level 1)

Purpose:

To determine the optimal chromatin:antibody ratio for ChIP.

Experiment summary:

Test 3 two-fold serial dilutions of human and mouse chromatin starting with 1 million cells per ChIP. Two antibody concentrations will be tested for each chromatin dilution. IgG negative control will be included in this part of the study. This is a total of 31 IPs.

Total number of IPs for level 2 validation for human: 31

Antibody concentration #1 to test: 3µg (human); 2.5 ug (mouse)
Antibody concentration #2 to test: 1µg (human); 5 ug (mouse)

Mouse chromatin ID: ID.1.13.131
Human chromatin ID: JM 12.14.120_MAST60

Procedure using Diagenode iDeal ChIP-seq kit (C01010050):

Protein A Magnetic immunoprecipitation: Day 1 (about 2 hours)

use siliconized 1.5ml eppendorf tubes and filter tips for each step

1. Take the required amount of DiaMag Protein A-coated magnetic beads (20 µl/IP). You will need 1320 µl.

2. Make a 12,000 µl 1x ChIP buffer iC1:
   Dilute 2,400 µl of 5X iC1 buffer with 9,600µl of ChIP grade water in a 15ml conical tube
   Place the diluted ChIP buffer iC1 on ice.

3. Wash the beads 4 times 2,640 µl of ice-cold 1x ChIP buffer iC1. For each wash, resuspend the beads by pipetting up and down several times and place the tubes in the 1.5 ml magnetic rack. Wait for one minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 3 times.

4. After the last wash, resuspend the beads in 1,320 µl 1x ChIP buffer iC1.

5. Prepare the ChIP reaction mix for 31 ChIPs + extra for error in a 15ml conical tube:

# of IP's	5% BSA (µl)	200x Protease inhibitor cocktail (µl)	5x buffer iC1 (µl)	Protein A Magnetic beads (µl)	ChIP-seq grade water (µl)
33	396	99	3696	1320	7690

6. **IMPORTANT*** Remove 400 µl and place in a separate tube. This is for IgG.

7. Aliquot the remaining ChIP working mix into **ten** 15ml conical tubes, one for each antibody concentration being tested. This should be 1200 µl per tube. Next, add in antibody and mix:

Antibody Code; Catalog	Antibody Concentration #1 (x 3 tubes)	Antibody Concentration #2 (x 3 tubes)
H3K36me3 (17); 61101	8 ug 17-1	4 ug 17-2

Add 4 uL of IgG to the IgG tube.

8. Aliquot 400µl of each antibody working mix into 3 tubes each (See table on next pg for tube labels).

1X Covaris shearing buffer (3.5 mls)
Add 350µl of 10X Covaris shearing buffer (D3) from kit (PN 520075) to 3150µl of cold diH2O plus 35 µl of 100X protease inhibitors.

HUMAN	Chromatin (10x10e6 cells/ml)	1X Covaris Shearing buffer	Total volume
1 million cells	2500µl	0	2500µl
0.5 million cells	1250µl	1250µl	2500µl
0.25 million cells	625µl	1875µl	2500µl
Mouse	Chromatin (10x10e6 cells/ml)	1X Covaris Shearing buffer	Total volume
1 million cells	700µl	0	700µl
0.5 million cells	250µl	250µl	500µl
0.25 million cells	125µl	375µl	500µl

9. Add *HUMAN* chromatin to the appropriate tubes:
****keep leftoever chromatin for input samples****

☐ To ***1*** tube of abs. conc. #1 __3__ µg, add 200µl of chromatin from ***1 million cells***.
☐ To ***1*** tube of abs. conc. #1___3__ µg, add 200µl of chromatin from ***0.5 million cells***.
☐ To ***1*** tube of abs. conc. #1 ___3__ µg, add 200µl of chromatin from ***0.25 million cells***.

☐ To ***1*** tube of abs. conc. #2 ___1__ µg, add 200µl of chromatin from ***1 million cells***.
☐ To ***1*** tube of abs. conc. #2____1_ µg, add 200µl of chromatin from ***0.5 million cells***.
☐ To ***1*** tube of abs. conc. #2 ___1__ µg, add 200µl of chromatin from ***0.25 million cells***.

To ***1*** tube of IgG, add 200µl of chromatin from ***1 million cells***.

Total volume per tube should be 400µl

10. Add *MOUSE* chromatin to the appropriate tubes:
****keep leftoever chromatin for input samples****

- ☐ To ***1*** tube of abs. conc. #1 __2.5__µg, add 200µl of chromatin from ***1 million cells***.
- ☐ To ***1*** tube of abs. conc. #1__2.5_µg, add 200µl of chromatin from ***0.5 million cells***.
- ☐ To ***1*** tube of abs. conc. #1 _2.5__µg, add 200µl of chromatin from ***0.25 million cells***.

- ☐ To ***1*** tube of abs. conc. #2 __5__µg, add 200µl of chromatin from ***1 million cells***.
- ☐ To ***1*** tube of abs. conc. #2__5_µg, add 200µl of chromatin from ***0.5 million cells***.
- ☐ To ***1*** tube of abs. conc. #2 __5__µg, add 200µl of chromatin from ***0.25 million cells***.

To ***1*** tube of IgG, add 200µl of chromatin from ***1 million cells***.

11. Incubate the tubes overnight at 4°C under constant rotation.

12. Remove 40µl of the remaining chromatin **at each dilution** to a new tube. These will be used as the input samples. Place them at 4 degrees overnight. Freeze leftover chromatin in case we need to repeat any input samples.

Elution and Reverse Crosslinking: Day 2 (about 4hours)

13. Prewarm iE1 buffer in 37 C water bath.

14. Briefly spin the tubes at 1300RPM and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash buffer iW1. To wash the beads, add 700 µl of iW1, gently shake the tubes to resuspend the beads and incubate for 5 minutes on a rotating wheel at 4°C.

15. Repeat the wash as described above once with Wash buffer iW2, iW3 and iW4 using the same buffer volume, respectively.

16. Make the reverse crosslinking mix:
    -Add 26.4 ml of iE1 buffer
    - 528 ul of Proteinase K (20mg/ml - Invitrogen)

17. After removing the last wash buffer, add 800µl of reverse crosslinking mix to the beads.

    *****Input samples*****
    Add 780µl of reverse crosslinking mix to 40µl of chromatin for each dilution (a total of 3 inputs).

18. Incubate for 30 minutes at 55C in the thermomixer at 900 RPM.

19. Briefly spin the tubes and add 32 µl of iE2 buffer. Wrap parafilm around the lids of the tubes to seal and minimize evaporation. Incubate overnight in a thermomixer at 900rpm at 65°C.
    If in a rush, it is possible to incubate at 4 hours and still get a good yield.

DNA Purification with QIAGEN MinElute PCR Purification kit:
Day 3 (about 3 hours)

20. In a separate 15ml tube, add 3.5ml of PB buffer and 40µl of 3M NaOAC. If you are doing multiple samples, I recommend making one large batch.

21. Add the reverse crosslinked material to the tube and vortex.

22. Transfer the sample to the minelute column and spin at 500xg for 1min. Repeat until sample is gone. Remove flow through each time.

23. Add 750µl of PE buffer and spin at 500xg for 1 min.

24. Remove flow through and spin at 13,000RPM for 1 min to dry column.

25. Transfer column to a new 1.5ml tube and add 55µl of buffer C (from Diagenode kit) or use water. Add to all samples and inputs. Exception: add only 30µl to IgG and 105 uL to inputs.

26. Let the samples sit at RT for 5min and spin at 13,000RPM for 1 min.

Quantitative Real Time PCR: Day 3 or4 (about 1 hour)

Positive target primers: GAPDH (human); GAPDH (mouse)
Negative target primers: MAGI1 (human); GD Chr 6 (mouse)

27. Set up the following quantitative real-time PCR reaction for each primer set:

	1X Mix (µl)	22 X mix human positive	22 X mix human negative	22 X mix mouse positive	22 X mix mouse negative
Sybr green (Select master)	10	220	220	220	220
Primers (10µM)	**1 or 2	22 or 44	22 or 44	22 or 44	22 or 44
Water	4	88	88	88	88

28. Load 15µl of PCR reaction mix into at 96 well dish.

29. Add 5µl of reverse crosslinked DNA to each well.

30. Select the "Enrichment PCR" assay on the qPCR:

Antibody 17; Active Motif 61101
-5 min at 95C

-40cycles of:
30 sec at 95C
30 sec at 60C
30 sec at 72C

-Add in a melting curve step.

31. Save file as LG.AbVal.14.1_qpcr and JM.12.14.105_qpcr

Tapestation: Day 3 or 4 (about 20 minutes)

34. Add 2µl of HS D1000 buffer to 2µl of ChIP DNA into a separate 0.2ml tube

35. Vortex for 5 sec and spin briefly

36. Load the tapestation tape, tubes and tips and run samples according to the manual instructions.

37. Click on the "electrograph" icon and "scale to sample" icon. Then click on edit peaks and select the major peak on the electrograph. Finally, click EPG snapshot. Repeat this step for all samples.

38. Under the file menu, click "create report" and then click "add EPG thumbnails"

39. Save file as LG.AbVal.14.1_HTS and JM12.14.105_HTS

Broad Range Quant-it assay: Day 3 or 4 (about 20 minutes)

41. Make a working solution:
6,965ul of HS buffer
35ul of HS reagent

42. Add 190ul of working solution to 29 wells of a 96-well black plate.

43. Add 10ul of standard to each well. This should come out to 0ng, 5ng, 10ng, 20ng, 40ng, 60ng, 80ng, 100ng.

44. Add 5ul of sample to each well.

45. Mix all wells by pipetting.

46. Run on plate reader. Choose protocol "picogreen". While on the plate layout, go to template editor and assign the standards and the unknowns (undiluted).

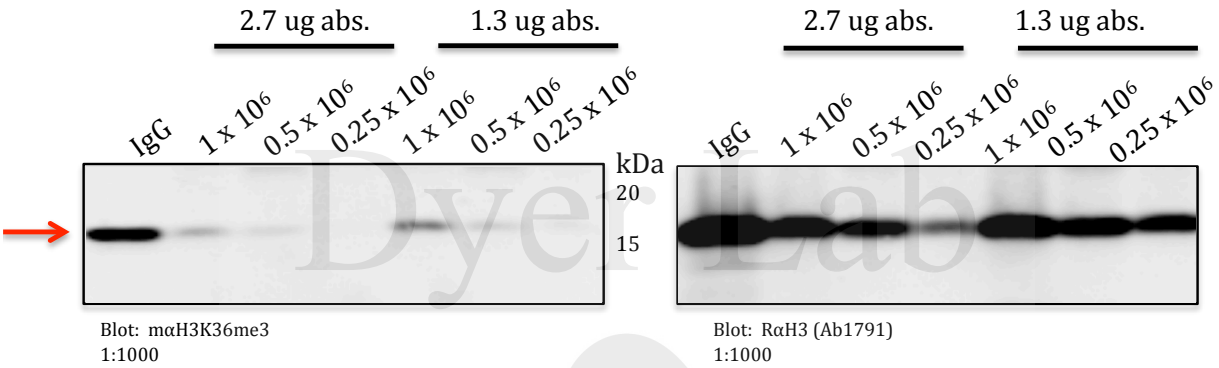
47. Save file as LG.AbVal.14.1_quantit and JM12.14.105_quantit.

Results:

Please copy and paste results. Do not type data into tables

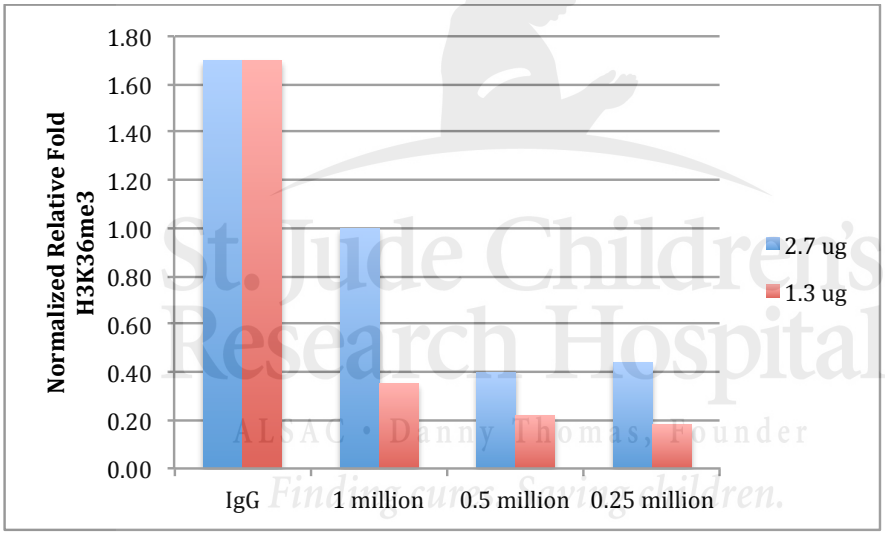
Protein bound to beads:

Human
ChIP: H3K36me3 (Active Motif 61101)



Blot: mαH3K36me3
1:1000

Blot: RαH3 (Ab1791)
1:1000



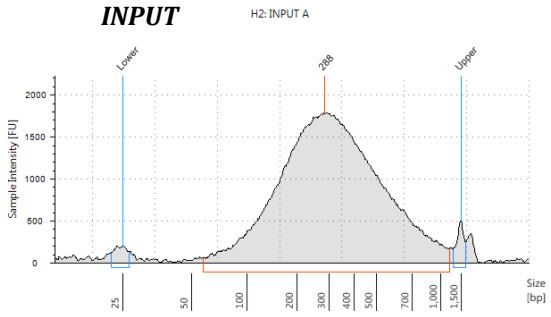
Date: 7/23/14 Experiment #: LG.Ab Val.14.1/JM12.14.105

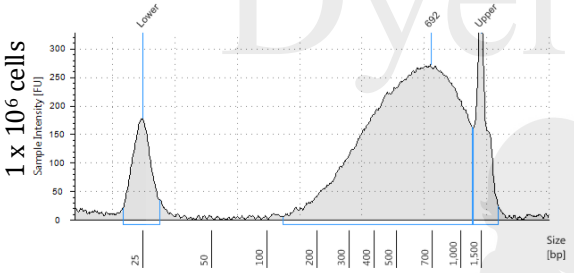
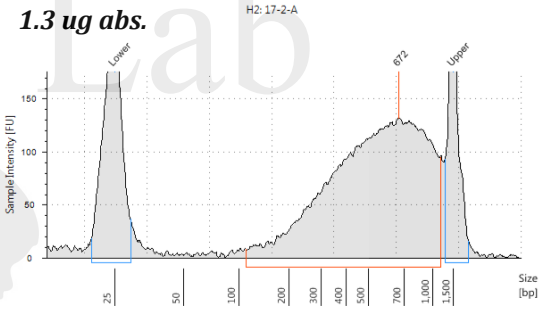
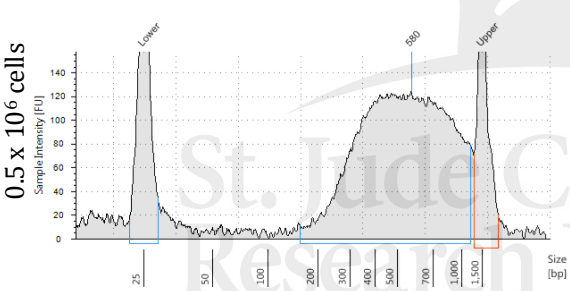
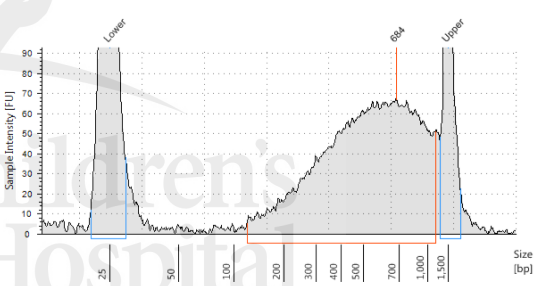
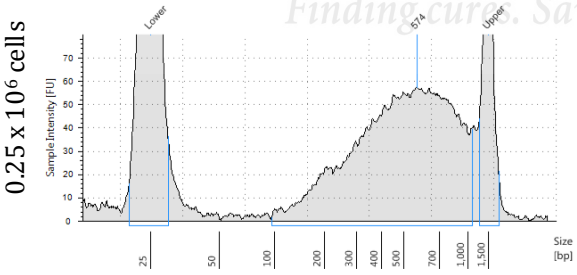
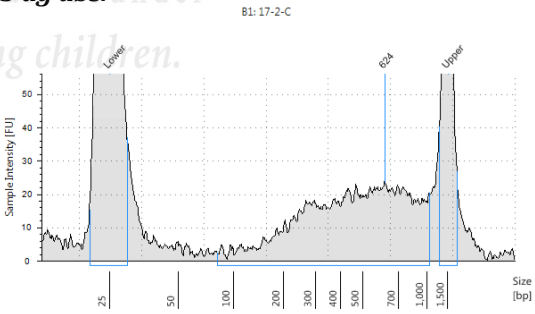
Antibody 17; Active Motif 61101

Bioanalyzer of ChIP DNA:

Human

INPUT



	2.7 ug abs.	***1.3 ug abs.***
1 x 10^6 cells		
0.5 x 10^6 cells		
0.25 x 10^6 cells		

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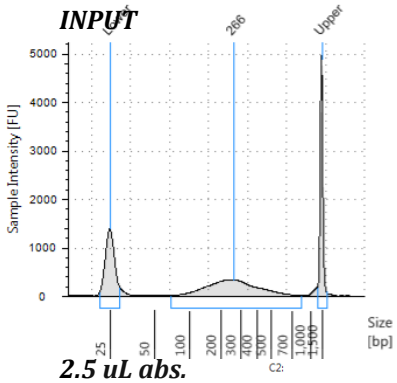
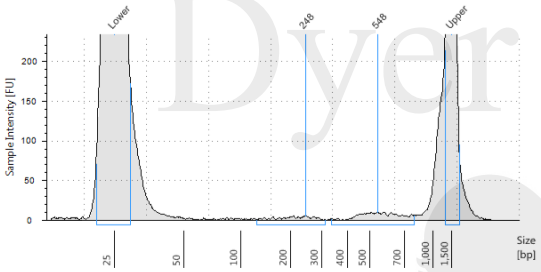


1 x 10^6 cells

2.5 uL abs.



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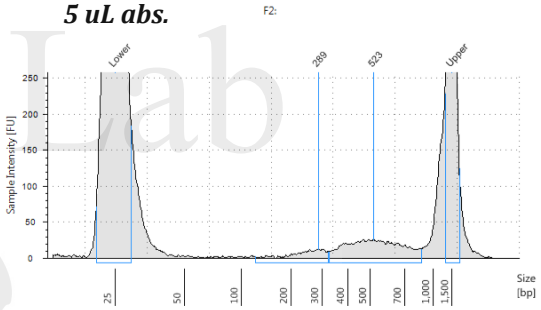
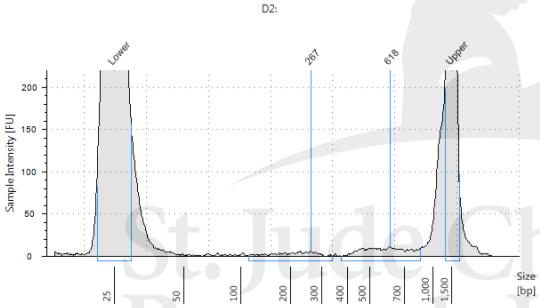
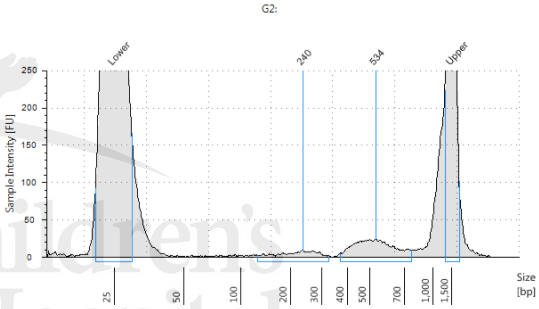


0.5 x 10^6 cells

2.5 uL abs.



5 uL abs.



0.25 x 10^6 cells

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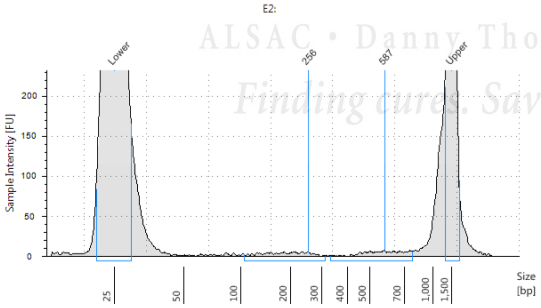
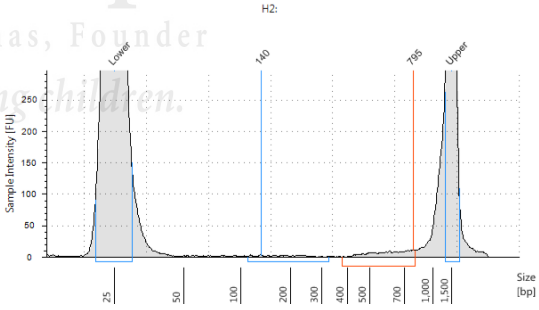


5 uL abs.



Date: 7/23/14 Experiment #: LG.Ab Val.14.1/JM12.14.105
Antibody 17; Active Motif 61101

Quant-it

HUMAN	**2.7µg ab.** (ng/µl)	**Total ng**		**1.3µg ab.** (ng/µl)	**Total ng**		**Input** (ng/µl)	**Total ng**		**IgG** (ng/µl)
1 million cells	3.95	197.42		1.60	79.95		22.65	1132.53		0.03
0.5 million cells	1.54	76.91		0.91	45.59		11.68	583.95		
0.25 million cells	0.77	38.68		0.34	16.77		5.60	280.06		
MOUSE	**2.5µg ab.** (ng/µl)	**Total ng**		**5µg ab.** (ng/µl)	**Total ng**		**Input** (ng/µl)	**Total ng**		**IgG** (ng/µl)
1 million cells							9.316	372.64		-0.51
0.5 million cells							3.5246	140.984		
0.25 million cells							0.9818	39.272		

Quantitative PCR

Positive target primers: GAPDH
Negative target primers: MAGI1

HUMAN

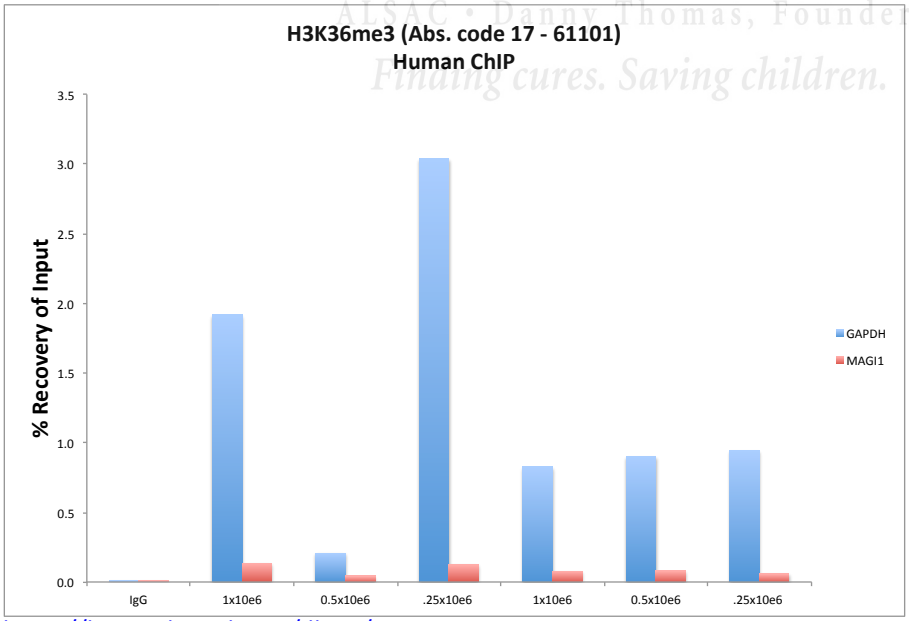
		GAPDH-1	**Average IP CT**	**% of input**	**MAGI1**	**Average IP CT**	**% of input**
2.7ug	1x10e6 cells	23.96	24.04	1.92	28.92	29.14	0.13
2.7ug		24.12			29.35		
2.7ug	.5x10e6 cells		28.33	0.21	33.12	31.74	0.04
2.7ug		28.33			30.36		
2.7ug	.25x10e6 cells	25.41	25.46	3.04	30.90	31.17	0.13
2.7ug		25.50			31.44		
		GAPDH 1	**Average IP CT**	**% of input**	**MAGI1**	**Average IP CT**	**% of input**
1.3ug	1x10e6 cells	25.20	25.26	0.83	29.85	29.92	0.08
1.3ug		25.31			29.98		
1.3ug	.5x10e6 cells	26.23	26.22	0.90	30.71	30.81	0.08
1.3ug		26.20			30.91		
1.3ug	.25x10e6 cells	27.00	27.14	0.95	32.01	32.25	0.06
1.3ug		27.28			32.48		
		GAPDH 1	**Average IP CT**	**% of input**	**MAGI1**	**Average IP CT**	**% of input**
2ug	IgG 1x10e6	40.00	40.00	0.00	40.00	40.00	0.00
2ug		40.00			40.00		
		GAPDH	**Adjusted CT**	**Average Input**	**MAGI1**	**Adjusted CT**	**Average Input**
	Input 1x10e6	21.64	18.32	18.34	22.93	19.61	19.60
		21.67	18.35		22.91	19.59	
	Input 0.5x10e6	22.75	19.43	19.42	23.89	20.57	20.56
		22.72	19.40		23.87	20.55	
	Input 0.25x10e6	23.70	20.38	20.42	24.82	21.50	21.53
		23.77	20.45		24.88	21.56	

Date: 7/23/14 Experiment #: LG.Ab Val.14.1/JM12.14.105
Antibody 17; Active Motif 61101

Positive target primers: GAPDH1 (14)
Negative target primers: GD Chr6 (13)

MOUSE

		Positive	**Adjusted CT**	**% of input**	**Negative**	**Adjusted CT**	**% of input**
2.5 ug	1x10e6 cells	28.28	28.28	0.96	30.32	30.38	0.21
2.5 ug		28.28			30.43		
2.5 ug	.5x10e6 cells	28.61	28.59	1.94	30.69	30.55	0.47
2.5 ug		28.57			30.41		
2.5 ug	.25x10e6 cells	29.59	29.55	2.70	31.37	31.50	0.68
2.5 ug		29.51			31.63		
		Positive	**Adjusted CT**	**% of input**	**Negative**	**Adjusted CT**	**% of input**
5ug	1x10e6 cells	27.25	27.28	1.92	28.85	28.86	0.60
5ug		27.31			28.86		
5ug	.5x10e6 cells	28.51	28.52	2.04	30.12	30.15	0.62
5ug		28.52			30.17		
5ug	.25x10e6 cells	30.91	30.73	1.20	32.02	32.02	0.48
5ug		30.54			32.02		
		Positive	**Adjusted CT**	**% of input**	**Negative**	**Adjusted CT**	**% of input**
5ug	IgG 1x10e6	35.08	34.82	0.01	33.62	33.70	0.02
5ug		34.55			33.77		
		Positive	**Adjusted CT**	**Avg. Input**	**Negative**	**Adjusted CT**	**Avg. Input**
	Input 1x10e6	24.89	21.57	21.58	24.78	21.46	21.47
		24.91	21.59		24.79	21.47	
	Input 0.5x10e6	26.24	22.92	22.90	26.16	22.84	22.81
		26.20	22.88		26.10	22.78	
	Input 0.25x10e6	27.73	24.41	24.34	27.66	24.34	24.31
		27.59	24.27		27.59	24.27	

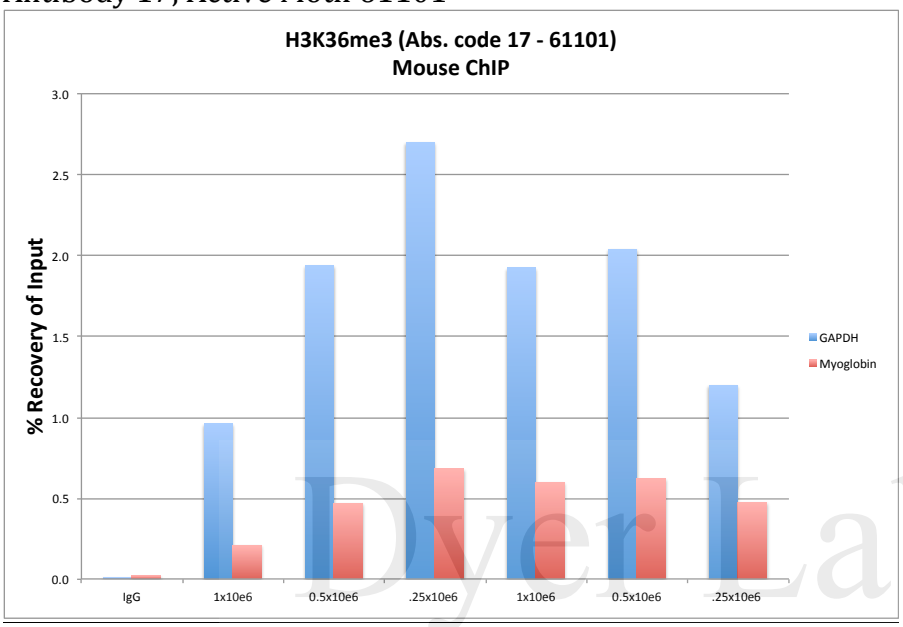


https://hospital.stjude.org/dbstp/

2.7µg abs. 1.3 ug abs.

Date: 7/23/14 Experiment #: LG.Ab Val.14.1/JM12.14.105

Antibody 17; Active Motif 61101



Conclusions: (provide yes or no answer)

Human Tissue

The ratio of the positive versus negative control targets is 5 or higher: NO

Antibody passes validation for level 2: YES

Mouse Tissue

The ratio of the positive versus negative control targets is 5 or higher: NO

Antibody passes validation for level 2: YES

Additional notes:

This antibody is recommended for use in ChIP-seq experiments for human tissue. I also recommend using 2-3 positive and negative target genes for qPCR.

I would recommend scaling up the ChIP reaction to make sure to get at least 10-15ng total DNA for submission to PCGP for ChIP-seq library prep.